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Review Paper

Decreasing soil microbial diversity is associated with decreasing microbial biomass under nitrogen addition

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ABSTRACT

While aboveground biodiversity has been widely studied, how microbial biodiversity responds to increasing nitrogen (N) deposition is still unclear. Here we conducted a meta-analysis to investigate the responses of soil microbial diversity and composition to N addition. Overall, we found N addition decreased both soil microbial diversity and the relative abundance of Actinobacteria and Nitrospirae, although the effect may vary among different ecosystems. The effect size on microbial Shannon index was positively correlated with the changes in soil microbial biomass under N addition. The initial soil conditions, the duration of treatment, the N addition rate and changes in soil organic carbon under N addition all affected the effect sizes of N addition on microbial Shannon index, while changes in soil pH played a minor role. Overall, our results suggest that the losses of microbial diversity with increasing N deposition rate would alter ecosystem functions and may have profound feedbacks to global climate change.

1. Introduction

Anthropogenic inputs of reactive nitrogen (N) have increased by two times since the industrial revolution (Vitousek et al., 1997; Galloway et al., 2008; Gruber and Galloway, 2008). While the increase of N deposition could relieve N limitation and increase net primary productivity in some ecosystems (Quinn Thomas et al., 2009), it may also cause negative ecological effects (Erisman et al., 2013). Our understanding of the ecosystem responses to increasing N deposition has mainly been limited to aboveground plant communities and soil nutrient cycling processes (Stevens et al., 2004; Xia and Wan, 2008; Bobbink et al., 2010; Lu et al., 2011; Simkin et al., 2016; Deng et al., 2017). Belowground soil microorganisms may also be sensitive to N availability, but have been much less studied. Soil microorganisms play an important role in nutrient cycling, soil organic matter decomposition, and other ecosystem functions and services (Brussaard, 1997; Van Der Heijden et al., 2008), and their responses to increasing N deposition may have serious consequences to global carbon (C) and N cycling and climate changes.

Recently, a few attempts of meta-analysis have been made to assess how microbial biomass would respond to N addition, and they found N addition generally suppresses microbial biomass (Treseder, 2008; Liu and Greaver, 2010; Zhou et al., 2017), but the effects may vary among different ecosystems (Geisseler et al., 2016). However, no meta-analysis is available to reveal the general responses of soil microbial diversity and composition to N addition. Microbial diversity and composition are key determinants of their ecological functions (Brussaard, 1997; Nannipieri et al., 2003; Philippot et al., 2013). Soil microbes are also potential resources of antibiotics and other drugs (Daniel, 2004). Besides, the diverse microorganisms have complex interactions among each other and have both redundant and specific functions. Therefore, microbial diversity and the related process level responses such as biomass, respiration rates, and enzyme activities should be examined simultaneously under the context of global changes.

Nitrogen addition could directly affect soil microorganisms due to the high osmotic potential and ion toxicity (Eno et al., 1955; Omar and Ismail, 1999) and the changes in N availability. On one hand, N addition may alleviate N limitation to some microbes, especially nitrifiers and denitrifiers who use inorganic N as their energy sources or electron acceptors. On the other hand, due to the high energy cost, N_2-fixing microbes may decline with increasing N availability (Berthrong et al., 2014) and microbes less tolerant to high osmotic potential may be killed, both resulting in the decline of microbial biodiversity.

Nitrogen addition could also indirectly affect soil microorganisms due to its influences on soil C availability, soil pH, and plant species richness (Zak et al., 2003; Treseder, 2008). For soil C availability, on

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one hand, N addition may increase net primary productivity (LeBauer and Treseder, 2008) and the quality of plant C (Liu et al., 2016b), which would result in the increase in labile C inputs into soils. Because soil microbes are mainly C limited (Fierer et al., 2009), the increase in labile C inputs is expected to increase microbial biomass. However, the biomass of different groups of microbes may all increase, resulting in unchanged microbial diversity. On the other hand, N addition may decrease fine roots production (Peng et al., 2017) and increase the fraction of recalcitrant compounds such as melanins and lignin (Liu et al., 2016b), resulting in less C availability to soil microbes. Therefore, microbial species specializing in the utilization of recalcitrant compounds may increase, while other species may decline or disappear. Furthermore, N addition has been found to decrease soil pH (Tian and Niu, 2015), enhancing the leaching of magnesium and calcium and mobilization of aluminum. Aluminum poisoning and magnesium and calcium limitation to microbes under N addition may also decrease the diversity of soil microbes and change microbial community composition (Bowman et al., 2008).

With the development of high-throughput sequencing technique, the determination of soil microbial community structure becomes more convenient and less expensive (Mardis, 2008). The number of studies on the effects of N addition on microbial diversity has increased lately, especially in the past five years (Allison et al., 2007; Freedman et al., 2015; Wang et al., 2015). A meta-analysis on these results is necessary in order to better understand the general pattern of N addition effects on soil microbial diversity. Additionally, knowledge on which microbial groups increase and which microbial groups decline under N addition could also help our understanding of the mechanisms of N addition effects on soil microbes. In this study, we used a meta-analysis approach to answer three questions: 1) How did soil bacterial and fungal diversity change under N addition? 2) How did the dominant phyla of soil microorganisms change under N addition? and 3) What are the underlying mechanisms of N addition effects on soil microorganisms? The Shannon index (Shannon, 1948) and Chao1 (Chao, 1984) were selected as our metrics for microbial diversity and richness because they are highly recommended when analyzing microbial alpha diversity (Lemos et al., 2011; He et al., 2013; Delgado-Baquerizo et al., 2016). Based on previous findings, we hypothesized that (i) N addition would decrease soil microbial diversity, and cause an overall reduction in microbial biomass; (ii) N addition would change the abundance of different functional groups; (iii) N addition would affect microbial richness (Chao1) more than Shannon diversity index due to the loss of some rare species with low tolerance to N additions; and (iv) Changes in soil organic carbon (SOC), soil N and soil pH under N addition treatments would drive the observed changes in soil microbial diversity.

2. Materials and methods

2.1. Selection criteria

To explore the effects of N addition on soil microbial diversity, we analyzed results from published data using a meta-analysis method (Hedges et al., 1999). We searched the online database (ISI Web of Science, Science Direct, Google and Google Scholar) using the following keyword combinations: “microbial diversity” or “alpha diversity” and “N addition” or “fertilization”. The following criteria were used to select appropriate studies: (1) Only field N addition studies were selected and laboratory incubation studies were not included; (2) At least one microbial diversity metric (Shannon or Chao1) was reported; (3) The N addition and control plots were established to have the same ecosystem types, dominant plant species, and soil types; (4) When studies reported data from several soil layers, only data from the upper 20 cm were included; (5) Treatments which differed in their N application rates and types of N fertilizers were entered as individual treatments. Some studies with treatments of different P or K fertilizers were also included.

2.2. Data extraction

A total of 55 studies across the world met our criteria and were included in this meta-analysis (Fig. S1, Table S1). For each study in our database, we noted the experiment location (latitude and longitude), climatic factors (mean annual temperature and precipitation), ecosystem types, durations of experiment (years), nitrogen fertilizer types, nitrogen application rate (g N m^{-2} yr^{-1}), microbial diversity metrics (Shannon or Chao1) and relative abundance of major soil microbial community members (ten major phyla for bacteria and two phyla for fungi). Data were extracted from tables and figures. The standard deviation was either reported or calculated from the standard error and sample size. When data were presented in the form of figures, they were extracted by Engauge Digitizer (Free Software Foundation, Inx., Boston, MA, USA). In addition, soil properties including SOC, soil total nitrogen (TN), C/N ratio, pH value, and microbial biomass carbon (MBC) were included if they could be obtained. For those studies (6 out of 55 studies), which did not report climate data, we used the coordinates of the study sites to extract climate data from the WorldClim database (http://www.worldclim.com) using ArcGIS (Version 10.0, ESRI, Redlands, CA).

Finally, we found a total of 198 data points, with 171 observations on Shannon index and 102 observations on Chao1 (Table S1). These data covered a wide gradient of climatic conditions and soil properties. For example, the altitude, mean annual temperature and precipitation ranged from 2 to 3500 m, from −7 to 20.6 °C, and from 164 to 1749 mm, respectively. Soil properties such as SOC (0.19–45.7%), C/N ratio (5.8–24.7) and pH (4.0–9.0) also showed wide ranges. The duration of the N addition ranged from 1 to 110 years, averaging 15 years. The annual N application rate ranged from 0.001 to 80 g N m^{-2}, averaging 13.7 g N m^{-2}. In addition, urea and ammonium nitrate (NH_4NO_3) were the most commonly used fertilizers. The fertilizer combinations included in our database were N alone (66.3%), NP (12.6%), NK (2%) and NPK (19.1%). The microbial analysis methods were generally divided into two groups: sequencing (72%, including Roche 454, Illumina MiSeq, Illumina HiSeq, Mega BACE and PacBio RS) and molecular fingerprinting (28%, including denaturing gradient gel electrophoresis (DGGE) and terminal-restriction fragment length polymorphism (T-RFLP)).

2.3. Statistical analyses

Meta-analyses were conducted on microbial diversity (Shannon index, H) and richness (Chao1), which were calculated using the following equations:

$$\text{Shannon index (H)} = -\sum_{i=1}^{s} p_i \ln p_i \quad (1)$$

where p_i is the proportion (n/N) of individuals of one particular species found (n) divided by the total number of individuals found (N), and S is the number of species.

$$\text{Chao1} = S_{obs} + \frac{F_1^2}{2F_2} \quad (2)$$

where S_{obs} is the total number of species observed in a sample; F_1 is the number of singleton species and F_2 is the number of doubleton species. Chao1 represents microbial richness, while Shannon index considers both richness and the relative abundance of different groups. Therefore, Chao1 is more sensitive to rare species in the community. It could be possible that Shannon index increases while Chao1 decreases under the same treatment, which generally would suggest potential loss of rare species.

The response ratio statistic was used to estimate effect size (Hedges et al., 1999). The response ratio ($\log_e R$) is the difference between the mean $\log_e$-transformed treatment value ($\overline{X}_t$) and the mean $\log_e$-transformed control value ($\overline{X}_c$):

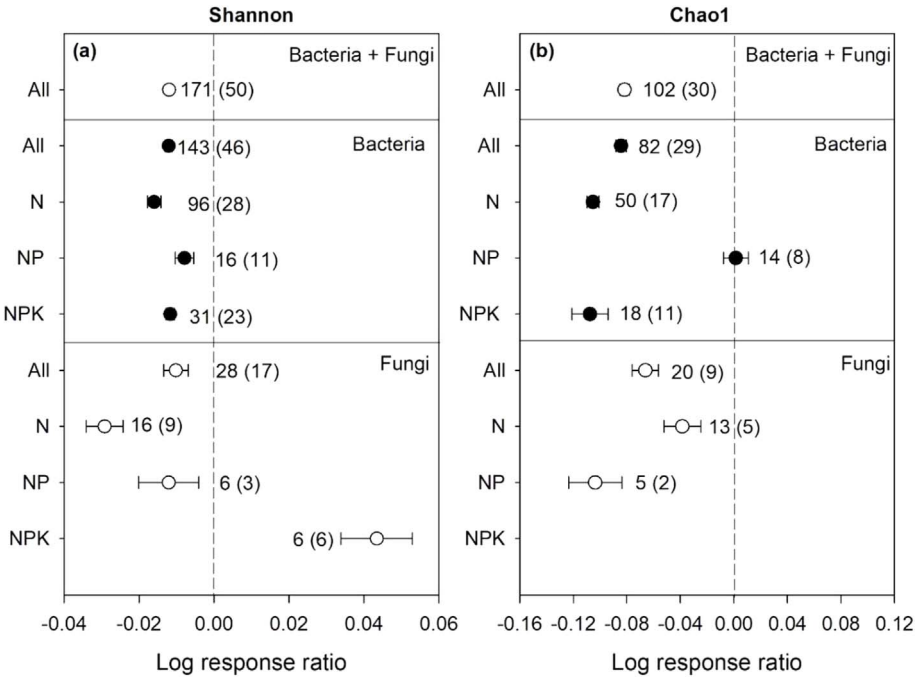

Fig. 1. Effects of nitrogen (N) addition on soil microbial Shannon index (a) and Chao1 (b). The category of 'All' includes studies from N addition alone, N + phosphorus (P) addition and N + P + potassium (K) addition. Error bars present 95% confidence intervals (CIs). The numbers next to the point represent the number of observations and studies (in parentheses).

$$\log_e R = \log_e\left(\frac{\overline{X}_t}{\overline{X}_c}\right) \quad (3)$$

The variance (v) of $\log_e$R was approximated using the following formula:

$$v = \frac{s_t^2}{n_t \overline{X}_t^2} + \frac{s_c^2}{n_c \overline{X}_c^2} \quad (4)$$

where s_t and s_c are the standard deviations for the treatment and control groups, respectively; n_t and n_c are the sample sizes for the treatment and control groups, respectively.

A nonparametric weighting function was used to weight individual studies (Hedges et al., 1999; Bai et al., 2013). For each study, the weighting factor w was calculated as the inverse of the pooled variance ($1/v$). When multiple observations were extracted from the same study, we adjusted the weights by the total number of observation per site (n). The final weight (w') used in the analyses was:

$$w' = \frac{1}{vn} \quad (5)$$

Then we obtained a weighted effect size $\log_e R'$:

$$\log_e R' = w' \times \log_e R \quad (6)$$

Finally, the mean variance-weighted effect size ($\overline{\mathrm{Log}_e R'}$) for all observations was calculated using the following formula:

$$\overline{\log_e R'} = \frac{\sum_i \log_e R_i'}{\sum_i w_i'} \quad (7)$$

Where $\log_e R_i'$ and w_i' are $\log_e R'$and $w'$of the ith observations, respectively.

We used MetaWin software to determine if experimental N addition had a significant effect on microbial diversity (Rosenberg et al., 2000). For each mean effect size, 95% confidence intervals (CIs) were calculated. If the 95% CIs of $\overline{\log_e R'}$ did not overlap with 0, then responses were significant at $P < 0.05$. We first examined the effects of different fertilizer combinations (N, NP and NPK) on soil diversity changes. Then we compared responses of microbial diversity and richness under N addition alone (N) among ecosystem types (cropland, grassland, forest, shrubland/desert, and tundra), fertilizer types (NH_4NO_3, urea, NO_3^- and NH_4^+), soil microbial analysis methods (DGGE, T-RFLP, Illumina MiSeq and Roche 454), nitrogen fertilization rate (< 5, 5–10, and > 10 g N m^{-2} yr^{-1}) and experiment duration (< 5, 5–10, and > 10 years). We also explored whether different soil pH (≤7 and > 7), C/N ratio (≤10 and > 10), and SOC (≤20 g kg^{-1} and > 20 g kg^{-1}) in control sites (representing initial soil conditions before treatments) affected the effect of N addition on Shannon index.

In addition to the variance-weighted meta-analyses, unweighted meta-analyses that included effect size without associated variances were applied to assess overall effects of N addition on the relative abundance of major bacteria and fungi groups at the phylum level.

A general linear model (GLM) was used to evaluate the relationship between the effect size ($\log_e R'$) on Shannon index and the effect size on soil microbial biomass (MBC). We further used structure equation modeling (SEM) to explore primary driving factors of effect size ($\log_e R'$) of N addition on microbial Shannon index. The duration of experiment (years), N addition rate (g N m^{-2} yr^{-1}), and the effect size of soil properties ($\log_e$ ($X_{treatment}/X_{control}$)), including SOC, TN, C/N ratio, pH value, and MBC were selected as the driving factors. We started the SEM procedure with the specification of a conceptual model of hypothetical relationships, based on *a priori* and theoretical knowledge. In the SEM analysis, we compared the model-implied variance-covariance matrix with the observed variance-covariance matrix. Data were fitted to the models using the maximum likelihood estimation method. Since some of the variables introduced were not normally distributed, the probability that a path coefficient differs from zero was tested using bootstrap (Grace, 2006). For simplicity, the least significant path was deleted and the model was re-estimated; then the next least significant path was removed, and so on, until the paths that remained in the final SEM were all significant. The chi-square test (χ^2) was used to examine the overall goodness of fit for SEM. When the model fit index of χ^2/df is within 0.00–2.00 and p value larger than 0.50, the SEM was considered acceptable (Schermellehengel et al., 2003). We also calculated the net influence that one variable has upon another by summing all direct and indirect pathways between the two variables. All the SEM analyses were conducted using AMOS 20.0 (Amos Development Company, Crawfordville, Florida, USA).

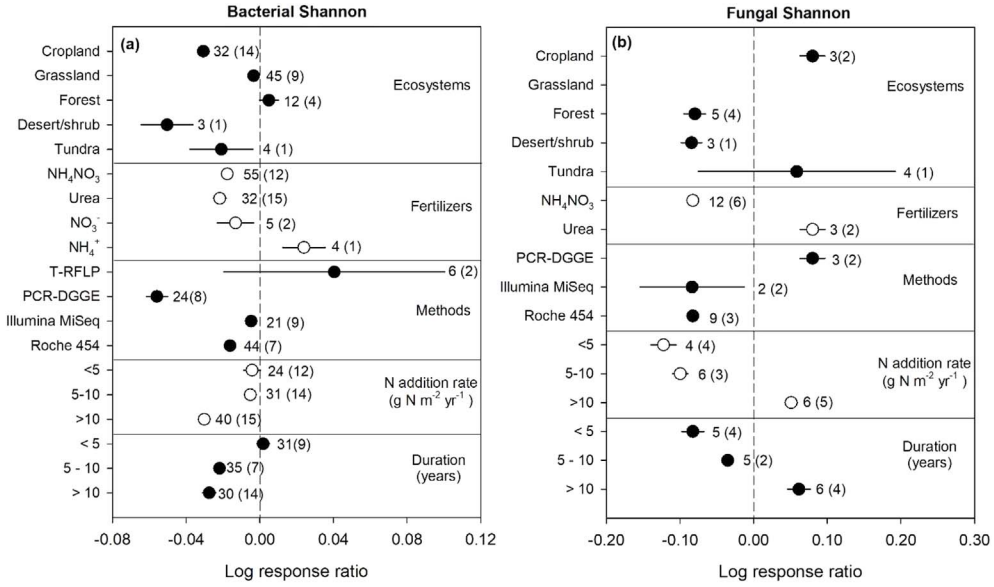

Fig. 2. Effects of nitrogen (N) addition on Shannon index of soil bacteria (a) and fungi (b). The variables are categorized into different ecosystems, fertilizer types, microbial analysis methods, N application rates and experiment durations. Error bars present 95% confidence intervals (Cls). The dashed line was drawn at mean response ratio = 0. If 95% CI does not overlap zero, then microbial diversity in fertilized soils differed significantly from those in unfertilized soils. The numbers next to the point represent the number of observations and studies (in parentheses). The N addition treatments combined with the addition of other nutrients were excluded in this analysis.

3. Results

3.1. Nitrogen addition effects on soil microbial Shannon index

Across all the studies, the mean effect size of N addition on soil microbial Shannon index was -0.012 (95% CI: -0.013 — -0.011), showing a significantly negative effect of N addition on microbial diversity (Fig. 1a). For bacterial Shannon index, all N fertilizer combinations decreased Shannon index with an mean effect size of -0.016 (95% CI: -0.018 — -0.014). When other nutrients (P or PK) were added in addition to N fertilizer, the effect sizes became less negative. For fungal Shannon index, the overall effect size of N addition was -0.010 (95% CI: -0.014 — -0.007). The effect size under N addition alone (effect size = -0.029, 95% CI: -0.034 — -0.024) was larger than under NP (effect size = -0.012, 95% CI: -0.020 — -0.004). Especially, under NPK addition, fungal Shannon index was positive (effect size = 0.043, 95% CI: 0.034–0.053). Therefore, we used data of N addition alone for further analysis to test the microbial diversity responses to N addition under different experimental settings.

There were significant differences in effect sizes among ecosystem types ($p < 0.01$; Fig. 2a and Table S2). Nitrogen addition decreased bacterial Shannon index in all ecosystem types, with the effect size of -0.003 (95% CI: -0.006 — -0.001) in grasslands, -0.021 (95% CI: -0.038 — -0.004) in tundra and -0.050 (95% CI: -0.064 — -0.036) in desert/shrublands, except forest ecosystems which had an positive effect size of 0.005 (95% CI: -0.0001 — 0.010). The decrease of bacterial diversity was significant under NH_4NO_3, urea and NO_3^- treatments, but not under NH_4^+ treatment. Studies using T-RFLP method showed an increase in bacterial Shannon index under N addition, while studies using PCR-DGGE and sequencing methods (Illumina MiSeq and Roche 454) showed the opposite. The effect size of N addition on bacterial Shannon index significantly decreased from -0.004 (95% CI: -0.009 — 0.0003) under N application rate $< 5\,g\,N\,m^{-2}\,yr^{-1}$ to -0.030 (95% CI: -0.033 — -0.027) under N application rate $> 10\,g\,N\,m^{-2}\,yr^{-1}$. There were significant differences among different experimental durations ($p < 0.01$; Fig. 2a and Table S2), with higher effect size under treatment for > 10 years (effect size = -0.028, 95% CI: -0.031 — -0.024) than under treatment for 5–10 years (effect size = -0.022, 95% CI: -0.025 — -0.019) and < 5 years (effect size = 0.002, 95% CI: -0.002 — 0.005).

For soil fungi, N addition decreased fungal Shannon index in forest and desert/shrubland ecosystems with the effect sizes of -0.080 (95% CI: -0.095 — -0.065) and -0.084 (95% CI: -0.098 — -0.070), respectively, but increased fungal Shannon index with the effect size of 0.080 (95% CI: 0.063–0.097) in croplands (Fig. 2b). Fungal Shannon index decreased under NH_4NO_3 application, but increased under urea treatment. However, it should be noted that data under urea treatment was only from croplands. For different methods, the decrease in fungal diversity was significant in studies using Illumina MiSeq and Roche 454 methods. Moreover, the effect sizes of N addition on soil fungal Shannon index were showing an increasing trend from -0.123 (95% CI: -0.140 — -0.105) to 0.051 (95% CI: 0.043–0.059) with increasing N addition rate and from -0.083 (95% CI: -0.098 — -0.068) to 0.062 (95% CI: 0.046–0.077) with increasing experimental duration.

Initial conditions in control plots such as soil pH, C/N ratio, and SOC influenced the extent to which N addition affected microbial Shannon index (Fig. S2). While N addition tended to reduce microbial Shannon index (mean effect size = -0.015, 95% CI: -0.016 — -0.014) in soils with pH > 7 under control, it had no significant effect when background soil pH values were < 7. When soil C/N ratio in control plots was under 10, the decrease of microbial Shannon index was higher than that in soils with C/N ratio > 10. Moreover, the microbial Shannon index changed more in soils with SOC $> 20\,g\,kg^{-1}$ (mean effect size = -0.012, 95% CI: -0.013 — -0.011) than that in soils with SOC $< 20\,g\,kg^{-1}$ (mean effect size = -0.010, 95% CI: -0.011 — -0.008).

3.2. Nitrogen addition effects on soil microbial Chao1

Overall, soil microbial richness, as estimated by Chao1, decreased with an effect size of -0.082 (95% CI: -0.085 — -0.078) under N addition (Fig. 1b). For bacterial Chao1, N addition (all N fertilizer combinations) decreased Chao1 with an effect size of -0.105 (95% CI: -0.110 — -0.101). When nutrients (P and K) were added together with N, the effect sizes became larger (effect size = -0.108, 95% CI: -0.121 — -0.094). For fungal Chao1, the overall effect size of N addition (all N fertilizer combinations) was -0.066 (95% CI: -0.076 — -0.056). The effect size under NP addition (effect size = -0.104, 95% CI: -0.123 — -0.084) was more negative than that under N addition alone (effect size = -0.039, 95% CI: -0.052 — -0.025).

There were significant differences in effect sizes of N addition on Chao1 among ecosystem types (Fig. 3 and Table S3). For soil bacterial Chao1, the effect on Chao1 was generally the same as the effect on Shannon index in different ecosystems, except in forest where the effect size on Chao1 was negative (Fig. 3a and Table S2), while the effect size on Shannon index was positive (Fig. 2a). Nitrogen addition effect on

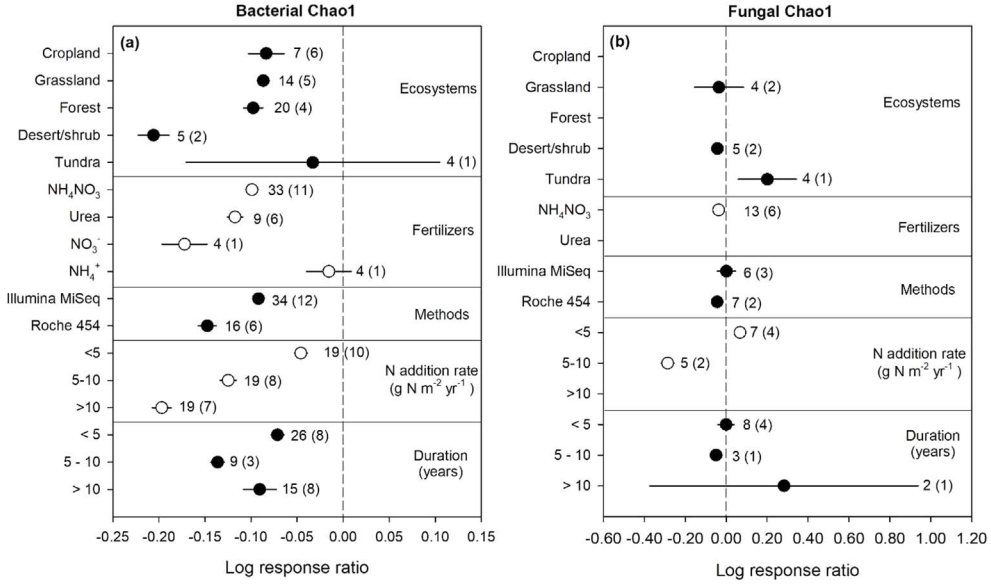

Fig. 3. Effects of nitrogen (N) addition on Chao1 of soil bacteria (a) and fungi (b). The variables are categorized into different ecosystems, fertilizer types, microbial analysis methods, N application rates and experiment durations. Error bars present 95% confidence intervals (CIs). The dashed line was drawn at mean response ratio = 0. If 95% CI does not overlap zero, then microbial diversity and richness in fertilized soils differed significantly from those in unfertilized soils. The numbers next to the point represent the number of observations and studies (in parentheses). The N addition treatments combined with the addition of other nutrients were excluded in this analysis.

soil bacterial richness was larger under treatment with NO_3^- fertilizer (mean effect size = -0.172, 95% CI: -0.197 — -0.148) than that under NH_4NO_3 (mean effect size = -0.099, 95% CI: -0.106 — -0.092) and urea (mean effect size = -0.118, 95% CI: -0.126 — -0.109) treatments. The effect sizes of N addition on soil bacterial richness became greater with increasing N addition rate (Fig. 3a). In addition, the bacterial richness changed most in experiments conducted for 5–10 years.

The number of observations was small (< 20) for the study of N addition effect on fungal Chao1 (Fig. 3b). The effect size of fungal Chao1 was positive in tundra under N addition (mean effect size = 0.201, 95% CI: 0.059–0.343), but was significantly negative in desert/shrublands (mean effect size = -0.044, 95% CI: -0.062 — -0.026) (Fig. 3b). When the N addition rate was < 5 g N m^{-2} yr^{-1}, the effect size of N addition on soil fungal Chao1 was 0.067 (95% CI: 0.043–0.091). In contrast, N addition significantly reduced fungal Chao1 when N addition rate was more than 5 g N m^{-2} yr^{-1}. In addition, N addition had no significant effect on fungal richness when the experimental duration was less than 5 years or more than 10 years, but had significant effect when the treatment duration was between 5 and 10 years.

3.3. Nitrogen addition effects on bacterial and fungal communities

At the phylum level, N addition significantly decreased the relative abundance of Acidobacteria (mean effect size = -0.185, 95% CI: -0.315 — -0.056) and Nitrospirae (mean effect size = -0.308, 95% CI: -0.590 — -0.026), but did not affect other examined bacterial groups (Fig. 4a). Neither of the major fungal groups Ascomycota and Basidiomycota significantly changed their relative abundance under N addition (Fig. 4b).

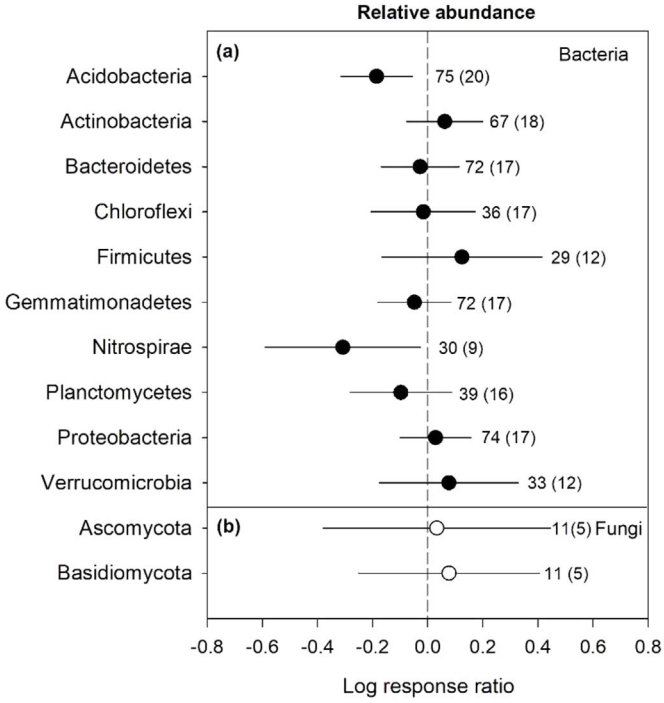

Fig. 4. Effects of nitrogen (N) addition on the relative abundances of soil bacteria (a) and fungi (b). Error bars present 95% confidence intervals (unweighted). The numbers next to the point represent the number of observations and studies (in parentheses). The N addition treatments combined with the addition of other nutrients were excluded in this analysis.

3.4. Relationships between microbial diversity and other factors under N addition

We found that the response ratio of microbial Shannon index to N addition was significantly correlated with the response ratio of soil microbial biomass (Fig. 5, $R^2 = 0.28$, $p < 0.001$). Moreover, the structural equation modeling (SEM) explained ~57% of the variance of microbial Shannon index under N addition (Fig. 6, $\chi^2/df = 1.38$, df = 16, $p = 0.14$). Duration of experiment and changes in soil organic carbon ($\log_e SOC_{treatment}$ - $\log_e SOC_{control}$) had positive direct effects on Shannon index, while N addition rate and soil C/N ratio ($\log_e C/N_{treatment}$ - $\log_e C/N_{control}$) had negative direct effects. The N addition rate also showed an indirect positive effect on microbial diversity via soil total N ($\log_e N_{treatment}$ - $\log_e N_{control}$). In addition, SOC and TN had indirect effects on Shannon index via C/N ratio. Overall, the duration of experiment and response ratio of total N had positive effects on microbial Shannon index under N addition while N addition rate, response ratio of SOC and response ratio of C/N ratio had total negative effects (Fig. S3). The changes in soil pH value had no effect on the change in microbial Shannon index under N addition.

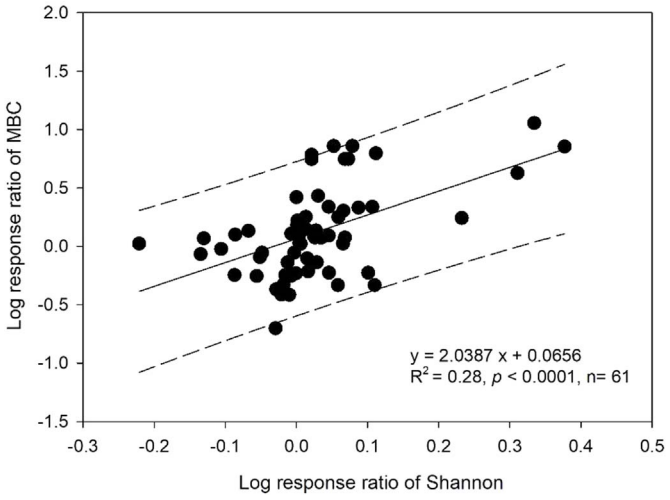

Fig. 5. Relationship between the response ratio of Shannon index and the response ratio of soil microbial biomass carbon (MBC) under N addition treatments including fertilizers with N and other nutrients. Dash lines present 95% prediction intervals.

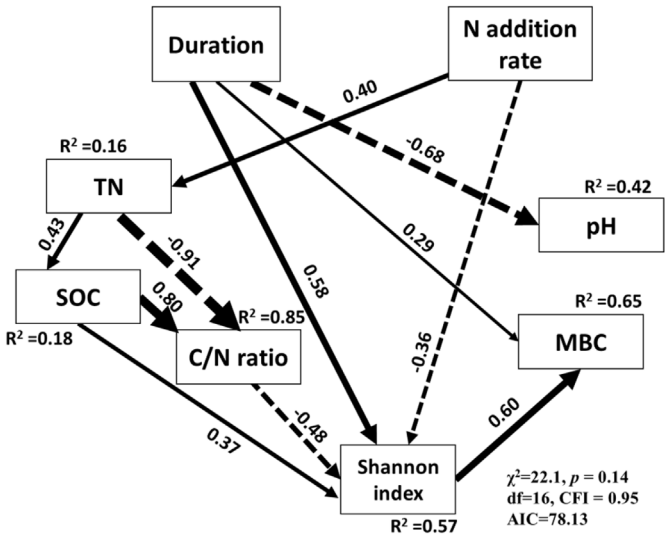

Fig. 6. Structural equational modeling (SEM) describing the effects of multiple drivers on the response ratio of bacterial Shannon index. Duration and N addition rate mean the experimental time (years) and the addition rate of fertilizer (g N m^{-2} yr^{-1}), respectively. SOC, C/N ratio, pH and MBC are the response ratios of soil organic carbon, soil C/N ratio, soil pH value and microbial biomass carbon under N addition. Continuous and dashed arrows indicate positive and negative relationships, respectively. R^2 denotes the proportion of response ratios of Shannon index explained by these drivers. Numbers adjacent to arrows are standardized path coefficients, analogous to relative regression weights, and indicative of effect size of the relationship. The thickness of the arrows is proportional to the magnitude of the standardized path coefficients or co-variation coefficients. χ^2, Chi-square; df, degrees of freedom; *p*: probability level; CFI: comparative fit index; non-significant χ^2 tests ($p > 0.05$) and CFI values over 0.90 are considered acceptable.

4. Discussion

4.1. Nitrogen addition generally reduces microbial biodiversity

The effect sizes of N addition on both Shannon index and Chao1 were negative, suggesting an overall decline of soil microbial biodiversity. We focused our discussion on bacterial diversity because fungal data were relatively insufficient to draw firm conclusions. The decreases in both bacterial Shannon index and Chao1 were most pronounced in desert/shrub ecosystems, pointing to the vulnerability of soil microbes in these ecosystems under the trend of increasing global atmospheric N deposition. Generally, Chao1 index decreased more than Shannon index under N addition in most studies (Fig. 1). Chao1 index presents species richness and is sensitive to changes in rare species while Shannon index accounts for both abundance and evenness of the species present in the sample (Shannon, 1948; Chao, 1984). Thus, the greater decrease in Chao1 under N addition suggested that rare species might have disappeared more. For example, N addition increased bacterial Shannon index in forest ecosystems, despite a decrease in Chao1, which suggested that although the evenness of soil bacteria increased, Chao1 in forests still declined due to the loss of rare species under N addition (Liu et al., 2016a; Cui et al., 2017). For different fertilizer types, NH_4^+ application caused an increase or no change in bacterial Shannon and Chao indexes, while other types of fertilizer decreased bacterial diversity. This result suggests that with the increasing proportion of nitrate in N deposition (Liu et al., 2013), the effects of increasing N deposition may become more severe due to the different effects of ammonium and nitrate on soil microbial diversity and biomass. However, observations on NH_4^+ treatment were all from one forest ecosystem, and whether the same phenomenon will happen in other systems should be further studied.

The effect sizes of both urea and NH_4NO_3 treatments on bacterial Shannon index were negative in both croplands and natural ecosystems (Table S5). However, urea treatment had positive effects on fungal Shannon index in croplands, while NH_4NO_3 had negative effects on fungal Shannon index in natural ecosystems. Therefore, the more positive effect of urea than NH_4NO_3 on fungal Shannon index might be ecosystem specific because most available observations were from croplands. More studies on fungal responses to N addition in more ecosystems are certainly needed to draw a more precise conclusion.

Although T-RLFP method showed different results from other methods for bacterial Shannon index (Fig. 2a), and PCR-DGGE method showed different results from other methods for fungal Shannon index, more than 70% of our data were based on sequencing and showed consistent results. Fingerprinting methods were believed to have lower accuracy compared with sequencing methods (Dowd et al., 2008; Cleary et al., 2012). Because microbial diversity indexes by fingerprinting methods tended to show positive or no response to N addition, our final conclusion of the negative effects of N addition was a conservative statement and the effect could be more negative if we used sequencing results only.

With increasing N addition rate, both Shannon index and Chao1 consistently showed a decreasing trend of bacterial biodiversity, indicating greater declines caused by the higher dose of N addition. Therefore, if N deposition keeps increasing in the future, the decline of bacterial diversity may become worse. It is interesting to see that fungal diversity showed different responses to higher N addition rate, in particular, fungal Shannon index even increased under N addition rate > 10 g N m^{-2} yr^{-1}. While the low number of data could be the reason of this inconsistency, it could also be possible that fungi respond differently from bacteria under high N addition treatment (Herold et al., 2012; Philippot et al., 2013; McHugh et al., 2017).

The addition of other nutrients (P, K) with N generally could mitigate the negative effects of N addition to some degree on both bacterial and fungal Shannon indexes, but had limited effects on bacterial and fungal Chao1. This result suggested that although the increase in soil other nutrients helped the resistance of some microbial groups to the negative effects of N addition, the abundance of rare species still declined, resulting in decreases in microbial richness. Therefore, regarding the decline in microbial diversity under N addition, the mitigation effect of other nutrients applied simultaneously with N was limited.

4.2. Nitrogen addition effects on microbial community structure

Although we could not tell which species disappeared under N addition by examining the changes in the relative abundances of major bacterial and fungal groups, we could detect the changes of microbial

community composition and gain a deeper understanding of the underlying mechanisms of N addition effects on soil microbes. For example, Acidobacteria surprisingly declined under N addition, which suggested that the decrease of soil pH was unlikely the mechanism of N addition effects on the decline of bacterial biomass because Acidobacteria are adapted to low pH conditions (Lauber et al., 2009; Shen et al., 2013). The SEM results provided additional evidence that pH changes had less influence on microbial community's changes under N addition. Our result also showed that N addition significantly decreased the relative abundance of Nitrospirae, although its relative abundance was very low in our database (0.1%–6.1%). The decrease of Nitrospirae indicated that nitrification activity may decline under N addition treatment.

4.3. Potential mechanisms of N addition effects on soil microbes

Nitrogen addition could directly affect soil microbes by changing soil inorganic N content or indirectly affect soil microbes by changing soil C availability, C:N ratio, and soil pH (Treseder, 2008; Serna-Chavez et al., 2013). So, we examined the relationships between the changes in these factors and the changes in bacterial Shannon index. First, higher SOC increased bacterial Shannon index although the total effect was very small. Therefore, the alleviation of C limitation to soil microbes under N addition would probably lead to a more diverse microbial community. Second, changes in soil pH did not affect bacterial Shannon index directly. Combining this result and the decrease in the relative abundance of Acidobacteria under N addition, we believe an increase in inorganic N instead of a decrease in soil pH caused the change in microbial diversity under N deposition, which is against our initial hypothesis (Lauber et al., 2009; Rousk et al., 2010). The increased soil inorganic N content may reduce the competition ability of microbes with less tolerance to high osmotic potential and N_2-fixing microbes (Kolb and Martin, 1988).

The different responses of soil microbes under different initial background conditions further confirmed the mechanisms of N addition effects on soil microbes. N addition had significantly larger effect on microbial diversity when N fertilizer was applied in alkaline soils than in acidic soils (Fig. S2). This result suggests the importance of considering the adaptation of soil microbes to the home field conditions when studying the responses of soil microbes to treatments (Goddard and Bradford, 2003). Furthermore, when soil C/N ratio was more than 10, N addition may somewhat mitigate the N limitation in soil microbes and the effect of N addition on microbial diversity would not be as severe as that in systems with sufficient N. Therefore, when studying the effects of N addition, the conditions before treatment may also affect the treatment results and should be carefully considered.

The significant positive correlation between the changes in bacterial Shannon index and the changes in bacterial biomass (Fig. 5) and SEM results (Fig. 6) both pointed to a link between microbial biodiversity and biomass, just like the aboveground plants (Grace et al., 2007; Humbert et al., 2016). Microbial communities have a central role in ecosystem processes by driving the Earth's biogeochemical cycles (Torsvik and Øvreås, 2002; Van Der Heijden et al., 2008) and thus the decline in microbial diversity and biomass under N addition would influence ecosystem processes and functions. First, microbial biomass residues have been identified as significant sources of soil organic matter (Simpson et al., 2007; Miltner et al., 2012). The decrease in soil microbial diversity and biomass may influence both the pool size and the chemical composition of soil carbon (Simpson et al., 2007; Liang et al., 2011). Second, N and phosphorus cycling may also be affected. Philippot et al. (2013) used a dilution approach to analyze the functional consequences of diversity loss and found that the reduction in denitrifier diversity resulted in a significantly lower potential denitrification activity. Besides, links between microbial diversity and plant productivity, plant diversity, or nutrient acquisition have also been observed (Zak et al., 2003; Van Der Heijden et al., 2008). Van Der Heijden et al. (2008) hypothesized that microbes and their diversity were likely to have the biggest influence on ecosystem performance in nutrient poor ecosystems, where small nutrient losses may immediately lead to a reduction of plant diversity. Therefore, the losses of microbial diversity with increasing N deposition rate would alter these ecosystem functions and may have profound feedbacks to global climate changes.

5. Conclusions

Overall, microbial diversity showed a robust decrease under N addition and this decrease was associated with the decline in microbial biomass. The changes in soil microbes under N addition were mainly due to the changes in soil nitrogen availability and soil organic carbon, while the role of soil pH was overall insignificant. With an increasing N deposition rate in the future, the decline in microbial diversity is likely to get worse, especially in desert and shrub ecosystems, although microbial acclimation may happen to some degree with longer duration of treatment. Background soil conditions before N addition should be considered to better understand and predict the effects of N addition on soil microbes. Fungal responses appeared to be different from bacterial responses to N addition and current studies on fungi were very limited, emphasizing the necessity of related studies in the future.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2018.02.003.

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